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Modified methods for efficiently differentiating human embryonic stem cells into chondrocyte-like cells*

Ulepszone metody różnicowania ludzkich embrionalnych komórek macierzystych w kierunku chondrocytów

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B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Summary

Introduction: Human articular cartilage has a poor regenerative capacity. This often results in the serious joint disease- osteoarthritis (OA) that is characterized by cartilage degradation. An inability to self-repair provided extensive studies on AC regeneration. The cell-based cartilage tissue engineering is a promising approach for cartilage regeneration. So far, numerous cell types have been reported to show chondrogenic potential, among others human embryonic stem cells (hESCs).

Materials and methods: However, the currently used methods for directed differentiation of human ESCs into chondrocyte-like cells *via* embryoid body (EB) formation, micromass culture (MC) and pellet culture (PC) are not highly efficient and require further improvement. In the present study, these three methods for hESCs differentiation into chondrocyte-like cells in the presence of chondrogenic medium supplemented with diverse combination of growth factors (GFs) were evaluated and modified.

Results: The protocols established here allow highly efficient, simple and inexpensive production of a large number of chondrocyte-like cells suitable for transplantation into the sites of cartilage injury. The most crucial issue is the selection of appropriate GFs in defined concentration. The obtained stem-derived cells reveal the presence of chondrogenic markers such as type II collagen, Sox6 and Sox9 as well as the lack or significantly lower level of pluripotency markers including Nanog and Oct3/4.

Discussion: The most efficient method is the differentiation throughout embryoid bodies. In turn, chondrogenic differentiation *via* pellet culture is the most promising method for implementation on clinical scale. The most useful GFs are TGF-β1, -3 and BMP-2 that possess the most chondrogenic potential. These methods can also be used to obtain chondrocyte-like cells from differentiating induced pluripotent stem cells (iPSCs).

Key words: **chondrogenesis • human embryonic stem cells • embryoid bodies • micromass culture • pellet culture • articular cartilage injury**

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Introduction

Articular cartilage (AC) degradation over the course of traumatic injuries, degenerative joint disease or congenital abnormalities is a common worldwide medical problem affecting people of all age groups. AC has a limited potential for self-repair and shows an insufficient healing response to injury [4,11,45].

Recently, stem cell-based therapy has become an attractive perspective in regenerative medicine and tissue engineering [21]. The term "stem cells" refers to an undifferentiated, self-renewing population of cells that are capable of differentiating to a desired cell lineage [17,39,40]. Currently, bone marrow-derived mesenchymal stem cells (MSCs) are good candidates for therapeutic applications. MSCs are adherent fibroblast-like cells with the capacity to differentiate into cells of different lineages, such as adipocytes, osteocytes or chondrocytes. Furthermore, the MSCs are easily isolated and, in contrast to mature chondrocytes, can be readily expanded *in vitro* to obtain a desired number of cells, which can then differentiate *ex vivo* [5,15,24].

The turning point in tissue engineering was the application of induced pluripotent stem cells (iPSCs) derived from human fibroblasts by transduction with transcription factors: Oct3/4 (*Octamer* Binding Transcription Factor *3/4)*), Sox2 (sex determining region Y-box 2), Klf 4 (Krüppel-like factor 4) and c-Myc. The iPSCs are capable of developing into derivatives of all germ layers and demonstrate immune compliance with a donor [18,31]. Currently, the similarities and differences between iPSCs and hESCs have been studied. It is believed that these two cell populations are equivalent in nearly all of their functions. However, they differ because of their distinct origins and modes of derivation [1,19,26].

The three principal methods of obtaining chondrocyte-like cells from ESCs that are suitable for transplantation into cartilage injury sites, embryoid body (EB) formation, the micromass culture (MC) and pellet culture (PC), are inefficient, time consuming, expensive and require modifications.

The aim of this study is to modify these methods and establish new protocols that allow for the efficient production of a large number of chondrocyte-like cells that are suitable for transplantation into the sites of cartilage injury.

Materials and Methods

Human embryonic stem cells (hESCs) culture

The hESCs BGV01 cell line (ATCC, VA, USA) was seeded onto 10 cm Matrigel (MG)-coated Petri dishes, with inactivated murine embryonic fibroblasts (MEFs) as a feeder layer ($1x10^6$ MEFs per dish). Briefly, 2 $x10^6$ cells were suspended in hESC growth medium supplemented with 10 ng/ml of fibroblast growth factor-2 (Merck Millipore, Germany) (Table 1) and seeded onto a single dish. The culture medium was changed daily.

Embryoid body (EB) formation

At 80% confluency, the hESC colonies were dissociated into clumps with a 0.25% trypsin/EDTA solution (Sigma Aldrich, MO, USA). The cells were then centrifuged to remove the trypsin/EDTA, resuspended in EB culture medium and transferred into non-adherent 96-well plates ($1x10^3$ cells per well) (Table 1). Within 24 h, free-floating aggregates of EBs were formed. The culture medium was changed every 48 h. At day 7, the EBs were used for chondrogenic differentiation.

Chondrogenesisin vitro

Two basic types of chondrogenic media were used: (i) supplemented with FBS (CM1) and (ii) serum-free (CM2) (Table 1). Prior to use, CM1 was supplemented with TGF-β3 (10 ng/ml) and CM2 was supplemented with the following growth factors: TGF-β1 (10 ng/ml) plus BMP-2 (100 ng/ml), BMP-2 (50 ng/ml) and BMP-4 (100 ng/ml or 50 ng/ml) (Immuno Tools, Germany).

Chondrogenesis *via* embryoid body (EB) formation

The mature EBs were transferred onto MG-coated 6-well plates (10 EBs per well) and allowed to adhere. After 24 h, the media was replaced with a chondrogenic media. The CM1 media was changed every 48 h while the CM2 media was changed daily. After 21 days of culture, immunofluorescence analysis was performed.

Table 1. Media composition

	Name	Composition
Growth Media (GM)	hESCs Growth Medium (hESCs GM)	DMEM F12 with L-Glutamine, 15% FBS, 5% KSR, 1% NEAA, 0.1 mM β-mercaptoethanol, 1% P/S
	EB Growth Medium (EB GM)	DMEM F12 with L-Glutamine, 15% FBS, 5% KSR, 1% NEAA, 0.1 mM β-mercaptoethanol, 1% P/S
	Differentiated Cells Growth Medium (DC)	DMEM F12 with L-Glutamine, 10% FBS, 1% P/S
Chondrogenic Media (CM)	Chondrogenic Medium 1 (CM1)	DMEM F12 with L-Glutamine, 10% FBS, 50 µM L-proline, 50 µM ascorbic acid, 1mM sodium pyruvate, 1% ITS+ Premix, 1% P/S, 10-7 M dexamethasone
	Chodnrogenic Medium 2 (CM2)	DMEM F12 with L-Glutamine, 1% KSR, 50 µM L-proline, 50 µM ascorbic acid, 1mM sodium pyruvate, 1% ITS+ Premix, 1% P/S, 10-7 M dexamethasone

DMEM F12, Dulbecco's modified Eagle's medium combined with Nutrient Mixture F12 with L-Glutamine and Hepes; DMEM HG, Dulbecco's Modified Eagle's Medium with high glucose; FBS, fetal bovine serum; KSR, Knockout Serum Replacement; NEAA, non-essential amino acids; P/S, Penicillin/Streptomycin; DMSO, dimethyl sulphoxide; ITS, Insulin-Transferrin-Selenium.

* CM, CM1 or CM2 medium depending upon variant of chondrogenic differentiation.

Chondrogenesis *via* micromass culture (MC)

The dissociated hESCs were cultured at a density of $1x10^7$ cells/ml in 10 spots (10 µl each) in (i) a6 cm culture dish, (ii) a 6 cm MG-coated culture dish (1:50) and (iii) a 6-well plate with a single 100 µl spot per well. The cells were suspended in chondrogenic media or in MG (1:50). The spots were incubated for 3 h to facilitate attachment. Next, the CM1 was added without dissociating the cell drops. The media was carefully changed every 48 h. After 21 days, immunofluorescence analysis was performed.

Chondrogenesis *via* pellet culture (PC)

Suspensions of hESCs in CM1 medium were distributed into 96 well round-bottom plates. Each well contained $2.5x\ 10^4$ cells suspended in 200 µl of CM1 media. The plates were centrifuged at 1200 rpm for 5 min. The media was changed every 48 h. After 21 days, immunofluorescence and immunohistochemistry analyses were performed.

Differentiated cell culture

The stem-derived cells were cultured on MG (1:50) in DC medium supplemented with FGF-2 (10 ng/ml) (Table 1).

Cryopreservation of differentiated cells

The differentiated cells were frozen in standard freezing medium (90% FBS+ 10% DMSO) or freezing hESC medium (hESCs GM+14% DMSO). After passage, the cells were suspended in 1 ml of proper freezing medium and transferred into cryotube vials. The vials were stored at -80°C for 24 h and then transferred into liquid nitrogen.

Immunofluorescence analysis

The cells or pellets were transferred into a MG-coated (1:50) 48-well plate for 24 h. Next, the media was replaced with a DC media. After 48 h, the cells were washed with PBS (phosphate buffered saline, Sigma Aldrich, MO, USA) and fixed for 20 min in 100% methanol (CHEMPUR, Poland), (400 µl of methanol per well). Then, the cells were rinsed with PBS containing 1% BSA (bovine serum albumin, Sigma Aldrich, MO, USA) and incubated for 30 min in PBS containing 1% BSA and 0.2% Triton X-100 (Sigma Aldrich, MO, USA). After 30 min, the cells were washed with PBS containing 1% BSA. The primary antibodies were diluted in PBS containing 1% BSA and 0.2% Triton X-100 and the cells were incubated overnight at 4°C with the following primary antibodies: type II collagen (Col2)(1:100), Sox9 (1:100), Sox6 (1:50), CXCR4 (1:100), chondroitin sulfate (1:100), E-cadherin (1:00) (all from Abcam, UK), Nanog (1:50) and Oct3/4 (1:50) (both from BD Biosciences, CA, USA).After conjugation with the primary antibodies, the cells were rinsed three times with PBS containing 1% BSA. The following secondary antibodies were diluted with 1% BSA in PBS and were incubated in the dark for 1 h at 37°C: mouse monoclonal anti-IgG, mouse monoclonal anti-IgM and rabbit polyclonal antibody (1:500) (Jackson ImmunoResearch, PA, USA). After washing three times with 1% BSA in PBS, the cells were stained for 5 min with diamidino-2-phenylindole dye (DAPI) (Sigma Aldrich, MO, USA) solution in water (1:10000) followed by washing with PBS and microscopic analysis.

Immunohistochemistry analysis

The histology specimens were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H&E) to visualize cellular content and subsequently analyzed under a light microscope.

Results

Differentiation via EBs

Differentiation *via* EBs was the most effective and technically simple method of chondrogenesis. Moreover, it

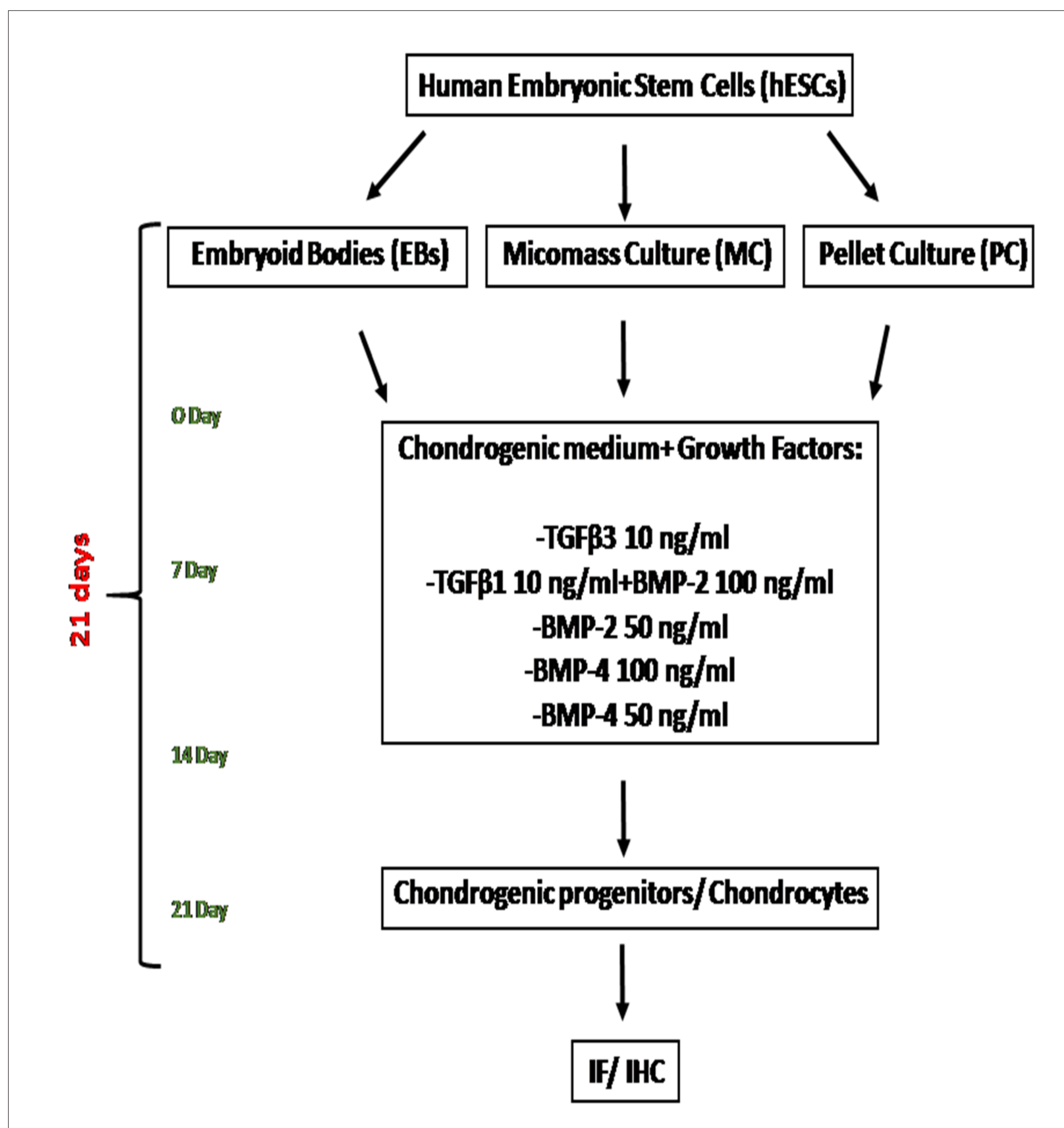

Fig. 1. The outline of the experiments conducted. Differentiation of human embryonic stem cells (hESCs) was performed for 21 days using three methods: embryoid bodies (EBs), micromass culture (MC) and pellet culture (PC). The chondrogenic media was supplemented with following growth factors (GFs): TGFβ3 (10 ng/ml), TGFβ1 (10 ng/ml) plus BMP-2 (100 ng/ml), BMP-2 (50 ng/ml), BMP-4 (100 ng/ml) and BMP-4 (50 ng/ml). The cells were evaluated by immunofluorescence (IF) or/and immunohistochemistry (IHC) analyses

allows us to observe morphological changes throughout the entire chondrogenic process. After attachment onto MG-coated plates, EBs were cultured in two different media: CM1 and CM2 (Fig. 2A).CM1 media was supplemented with TGF-β3 (10 ng/ml). This combination provided the best results. EBs acquired a much expanded morphology that was visible even with a naked eye. At the end of differentiation (approximately 17-21 days), this specific structure occupied the entire surface of a 6-well plate. During passage, the overgrown EBs were removed and the monolayers were used for analysis or further culture.

Differentiation in CM2 media gave distinct results depending on the added GFs. The most efficient growth was observed when TGFβ1 and BMP-2 were added at concentrations of 10 ng/ml and 100 ng/ml, respectively. The course of differentiation and the morphology of the cells resembled those treated with TGF-β3 (10 ng/ml) alone. Differentiation using BMP-2 (50 ng/ml) also

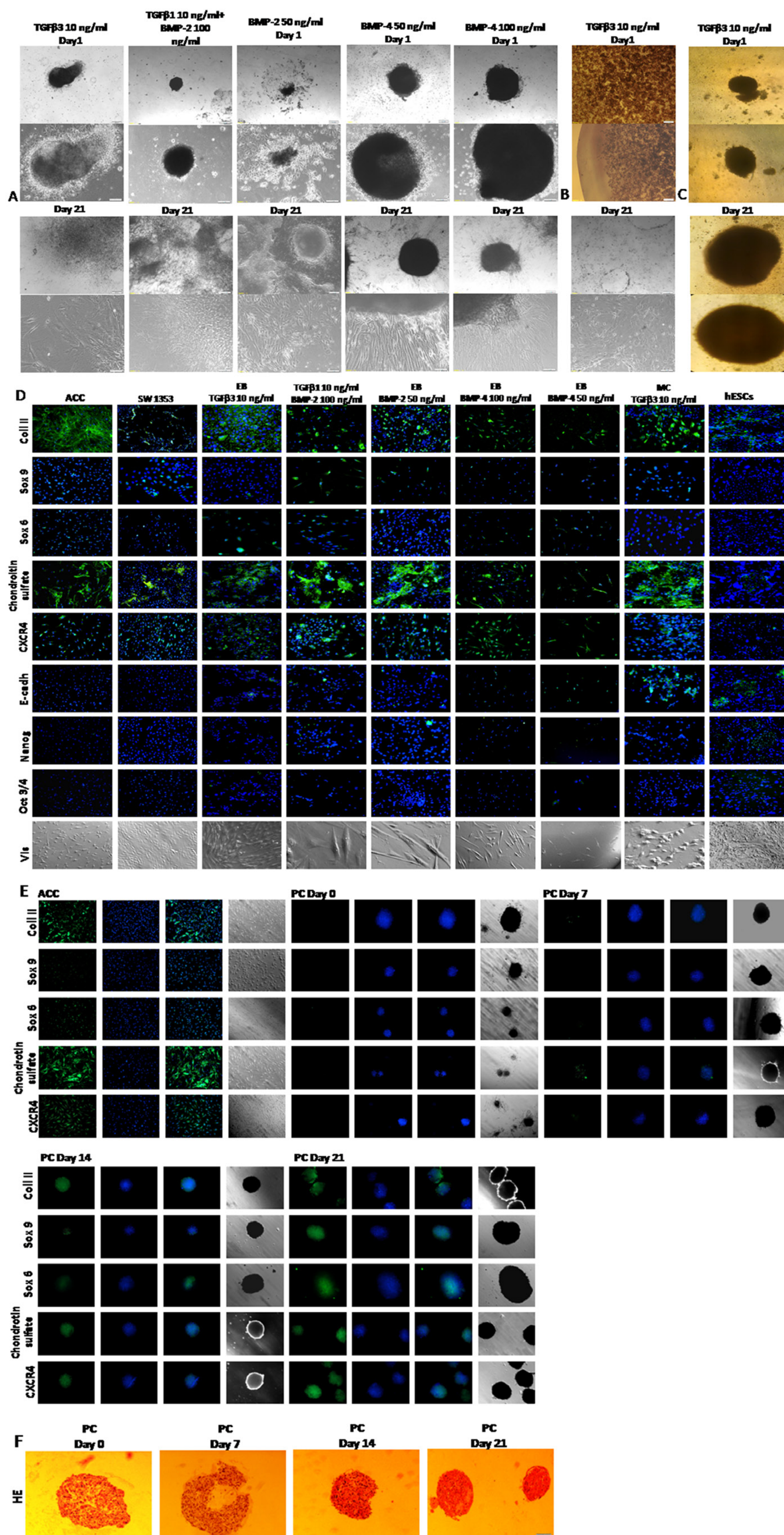

Fig. 2. During chondrogenic differentiation, cells assumed morphological features of human-like mature chondrocytes. The differentiantion was carried out for 3 weeks with the help of 4 methods: formation embryoid bodies (A), micromass culture (B) and C) pellet culture in the presence of selected growth factors. The differentiated cells revealed markers characteristic for articular chondrocytes such as: type II collagen, Sox6, Sox9, chondroitin sulphate, CXCR4 and decreased level of pluripotent markers: E-cadherin, Nanog and Oct3/4. As a positive control articular cartilage chondrocytes were used. Human embryonic stem cells served as a negative control (D). For precise examination of chondrogenesis in vitro, the cells during differentiation in pellet culture were investigated at 7 days by immunofluorescence (E) and immunohistochemistry (F) analyzes. In immunofluorscence evaluation were exploited following chondrogenic markers: type II collagen, Sox6, Sox9, chondroitin sulphate and CXCR4. As a positive control articular cartilage chondrocytes were used; hESCs - human embryonic stem cells, ACC- articular cartilage chondocytes, EB - differentiation throughout embryoid body formation, MC - differentiation throughout micromass culture, ACC - articular cartilage chondocytes, HE - hematoxylin and eosin staining

gave satisfying results. In this case, overgrown EBs and an expansion of proliferating cells could also be observed. Surprisingly, the EBs cultured in CM2 media supplemented with BMP-4 (50 ng/ml or 100 ng/ml) showed an almost identical morphology throughout the entire time of culture.

After 21 days, the cells were passaged. The cells cultured in CM1 media supplemented with TGF-β3 (10 ng/ml) were the most numerous and their proliferation rate was the highest. The proliferation rate was also elevated for cells cultured in CM2 with 50 ng/ml BMP-2. The cells cultured in CM2 media with TGFβ1 and BMP-2 (100 ng/ml and 50 ng/ml, respectively) revealed a moderate proliferation rate. The number of cells cultured in CM2 medium supplemented with BMP-4 (both 50 ng/ml and 100 ng/ml) was unsatisfactory and their proliferation rate was inferior.

Differentiation via MC

Chondrogenesis *via* MC gave ambiguous results. First, cells suspended in MG as regular drops and transferred into a well or plate quickly lost their uniform structure and detached from the surface. Second, the cells suspended in CM1 showed a higher growth rate depending on the surface and the size of the spot. However, it was very difficult to transfer the spots onto MG-coated plates because the spots immediately lost their regular structure. This problem was minimized when the spots were transferred onto wells without any scaffold. Another significant point was the size of drops. The 100 µl spots adhered remarkably better than the smaller ones (10 µl) (Fig. 2B).

Differentiation via PC

Differentiation *via* PC is the least complicated method of chondrogenesis. Immediately after centrifugation, the ESCs grew in CM1 media as a very thick suspension, but on the second day, they acquired a very similar size and morphology as the EBs (Fig. 2C). Interestingly, the cells created several small pellets apart from the main pellet. During chondrogenesis, the pellets increased in size. After 21 days, the pellets were used for further analyses.

Immunofluorescence and immunohistochemistry

Immunofluorescence was used to assess the differentiated cells. We took advantage of markers specific for articular cartilage chondrocytes, such as Col2, transcription factors Sox9 and 6, chondroitin sulfate proteoglycans as well as markers characteristic for pluripotent stem cells, E-cadherin, Nanog, and Oct3/4 (Fig. 2D). Additionally, chemokine receptor four (CXCR4) was also analyzed. In comparison to the positive control (primary human articular cartilage chondrocytes, ACC), all differentiated cells displayed expression of chondrogenic markers, although the level of their expression differed depending on the method used. The expression of Col2, a key chondrocyte marker, was the highest in cells from MC (CM1+TGF-β3, 10 ng/ml) and EBs (CM1+ TGF-β3, 10 ng/ml, and CM2+ TGFβ1, 10 ng/ml plus BMP-2 100 ng/ml). In turn, the expression of transcription factors Sox9 and 6 was the most apparent in cells differentiated *via* EBs in medium supplemented with TGF-β1 (10 ng/ml) plus BMP-2 (100 ng/ml). Chondroitin sulfate proteoglycans were present in all differentiated cells. CXCR4 expression was very similar in all cells after differentiation was completed. Nevertheless, the expression of CXCR4 was relatively low in the cells after MC. The immunofluorescence analysis demonstrated that, in comparison with the negative control (hESCs), E-cadherin was expressed by all cells at a low level, except the cells after MC. The pluripotent markers Nanog and Oct3/4 were expressed in the negative control, and very low or no expression was observed in the differentiated cells.

In the pellets, the chondrogenic markers: Col2, Sox9 and 6 and chondroitin sulfate as well as CXCR4 were observed. Their fluorescence signal increased proportionally to the culture period. The changes in marker expression were assessed at days 0, 7, 14 and 21 because of the limited capacity to observe their morphological changes during the culture period. It is noteworthy that Col2 expression was the most visible at day 14. In turn, the expression of other chondrogenic markers was the highest at day 21 (Fig. 2E). Hematoxylin and eosin staining indicated that necrosis was noticeable during the last week of differentiation (Fig. 2F).

Discussion

Chondrogenesisin vitro

In our study, hESCs were differentiated toward a chondrogenic lineage using embryoid body (EB) formation [36,37,38], micromass culture (MC) [7,10,43] and pellet culture (PC) [12,14] (Fig. 1). We modified and improved the protocols reported in the literature. To obtain feeder-free cells, the hESCs were seeded twice onto 0.5% gelatin-coated plates for 30 minutes, resulting in a feeder-free cell suspension. Chen et al. conducted the non-colony monolayer (NCM) cell culture in MEF-conditioned media, which eliminates the need for a MEF feeder layer and reveals the cytogenetic stability of cultured cells [6]. It is important to remember that chondrogenesis is a very complicated process that still requires further research. Thus, many factors such as positive influence of hypoxic conditions and various morphogenetic proteins on differentiation should be considered [22,46].

Chondrogenesisin xeno-free medium

We established xeno-free chodrogenic medium (CM2). Thus, the KSR (1%) instead of FBS (usually 10%) was used. It was validated that the chondrogenic process without animal-derived components can be successfully carried on. It is relevant in connection with Good Manufacturing Practice (GMP). According to the demands of

GMP, cell-based medical products at each stage of production, should be characterized by high quality and should meet very restrictive safety criteria [2,40]. Nowadays, it has a great importance since the tissue engineering is developing rapidly and in the near future stem cell-derived products will be used in the clinical practice. The investigations regarding pluripotent stem cell culture and differentiation in xeno-free conditions are intensively carried on [3,9,23,25,28,35]. Our results and literature data indicate that the xeno-free medium can be successfully implemented both in laboratory and clinical scale. The chondrogenic differentiation with the usage of xeno-free medium is as effective as chondrogenesis in the presence of chondrogenic medium with FBS.

Chondrogenesisvia EBs

We chose concentrations of the following transforming growth factor-β (TGF-β) superfamily members to favor early and advanced chondroge1nesis, but not hypertrophy leading to osteochondral ossification: TGF-β1, TGF-β3, BMP-2 and BMP-4. Our results revealed that TGFβ3, TGFβ1 and BMP-2 gave superior results in EB culture (Fig. 2A). They induced the expression of cartilage-specific markers: Col2 and Sox6 and 9 (Fig. 2D). Our results disclosed that TGF-β1 and BMP-2 exhibited chondrogenic effects alone or in combination. These results differ from previous reports. Toh and Cao claim that the combination of TGF-β1 and BMP-2 produces the most efficient induction of the chondrogenic marker, COMP. However, this was correlated with the lowest expression of Col2 and the highest expression of Col1, characteristic of osteogenesis [36]. This suggests that an overabundance of GFs may trigger cellular hypertrophy and, consequently, endochondral ossification and inhibition of cell development instead of chondrogenic induction. We observed a significantly lower level of proliferation in cells treated with TGF-β1 and BMP-2 after the first passage, than in those treated with BMP-2 alone, which may confirm Toh's hypothesis. In our findings, sequential administration of the growth factors gave very promising results. Handorf and Li selected the best GFs for differentiation of mesenchymal stem cells (MSCs) into chondrocytes. They examined hMSCs GF receptor expression every 3 days throughout differentiation and demonstrated that chondrogenesis was enhanced by the sequential administration of TGF-β1 and BMP-7, and showed that the lack of GFs between days 9 and 12 of chondrogenesis remarkably repressed the hypertrophic process [12]. This is likely to be important regarding chondrogenesis in hESCs and iPSCs.

Initially, EBs were differentiated on plates coated with MG. To reduce costs, MG was replaced by 0.1% gelatin with equally good results (data not shown). Our preliminary *in vivo* studies in a rabbit model confirmed that the differentiated cells had the capacity to fill the injury site (data not shown). However, further studies are required to determine whether the chondrocyte-like cells are fully functional or represent chondroprogenitors.

Chondrogenesis via MC and PC

The greatest obstacle of differentiation *via* EBs is the fact that this is not a direct method and is accompanied by an additional step of EB formation. Therefore, in further studies, we evaluated direct methods of chondrogenesis from hESCs *via* MC and PC in CM1 media supplemented with TGF-β3 (10 ng/ml) for 21 days (Table 2).

Table 2. Semi-quantitative evaluation of the differentiation protocols in relation to the most important parameters of cell culture

Parameter	Method		
	Embryoid Body (EB)	Micromass Culture (MC)	Pellet Culture (PC)
Number of cells initially required	low	high	high
Efficiency of differentiation	high	moderate	moderate
Overall cost	moderate	moderate	low
Time needed	long	intermediate	short
Application on clinical scale	moderate	moderate	high

The hESCs were differentiated in CM1 media supplemented with TGF-β3 (10 ng/mL)

The use of MG did not succeed because it is not suitable as a carrier of cells or as a scaffold in the MC culture. Although this method seems to be simple, it generates two main problems: an uncomfortable procedure for changing media and the differential adhesion of the drops to the plate surface. We partially resolved this problem by applying bigger spots (100 µl instead of 10 µl) and changed the medium very carefully in order to preserve the structure of drops. However, the application of this method might be problematic on clinical scale. These problems was overcome when plates with better adherent properties were used. Gong et al. carried out the differentiation of hESC *via* MC. The hESC were differentiated as 10 µl spots in serum-free medium containing BMP-2 (100 ng/ml), TGF-β1 (10 ng/ml) or both for 14 days. According to these authors, the cells did not undergo hypertrophic maturation and could be good candidates for repairing cartilage defects [10]. Diekman et al. made progress in the chondrogenic differentiation of pluripotent stem cells in MC media containing mouse BMP-4 (50 ng/ml). They differentiated mouse iPSCs and then purified the cells according to the presence of type II (Col2)- driven green fluorescent protein (GFP). They found that the GFP-positive cells showed a significantly higher level of Col2 and aggrecan than the GFP-negative cells, and their ability to repair cartilage injuries was improved [7]. This method seems to be very prospective because the immunofluorescence analysis revealed

that the heterogeneous cell population that we obtained consists of undifferentiated, partially differentiated and completely differentiated cells.

Yamashita et al. described chondrogenic differentiation of human pluripotent stem cells *via* MC. They used chondrogenic media containing 1% FBS and a combination of TGF-β (10 ng/ml) and BMP-2 (10 ng/ml) for the first 5 days, and BMP-4 (10 ng/ml) alone thereafter. Furthermore, they demonstrated the usefulness of this method for screening abnormal iPSCs, which are connected with oncogenic risk [43].

We also evaluated a method of direct hESC differentiation *via* PC. The best results, judging from the expression of chondrogenic markers as well as the lack of cell necrosis, were achieved at day 14, in contrast to the majority of reports recommending a three week culture. Nonetheless, there are some reports that chondrogenesis *in vitro* may last for a shorter period. The above-mentioned investigations of Gong et al. also applied a 14-day culture period.

After 21 days of culture, we noticed that necrotic areas are created in the centers of the pellets and that this phenomenon can be avoided by continuous stirring because it facilitates the interaction between the cultured cells and the components of the media [27,34]. The great advantage of PC is its simplicity. The cells in pellets are differentiated in conical tubes (15 or 50 ml), Eppendorf tubes or round-bottom plates, making it easy to scale-up the culture. Hwang et al. obtained chondrogenically committed cells from hESCs *via* co-culture with adult chondrocytes. Then, these cells underwent differentiation in pellets without the addition of GFs because the chondrocytes likely supplied the morphogenetic proteins necessary for differentiation. Nonetheless, PC supplemented with GFs exhibited a better expression of markers specific for articular cartilage. This is an example of feeder-free culture of embryonic stem cells [14]. Karlsson et al. established a protocol for the efficient differentiation of human ESC-derived mesenchymal progenitor cell lines. Afterwards, they successfully differentiated these cells into osteogenic, chondrogenic, and adipogenic lineages. This is a method of obtaining a homogenous, easy to culture, and rapidly proliferating population of cells that originate from the same germ layer. These cells do not require any coated plates, feeder layers or even conditioned media. In addition, this method ensures the GMP-grade production of chondrocytes [16]. A very similar attitude was adopted by Tanaka et al., who, through WNTs and BMPs control, generated ESC-derived osteochondrogenic mesodermal progeny having characteristics of mesoderm [33]. The production of mesodermal progeny from human iPSCs followed by sequential administration of chondrogenic factors was proposed by Umeda et al. who achieved chondrogenic differentiation *via* MC in 16 days using GF supplementation (40 ng/ml PDGF plus 10 ng/ml TGF-β3 for 3 to 6 days and 50 ng/ml BMP-4 from the tenth day on) [41]. A very advanced and promising research was demonstrated by Nejadnik and co-workers. They differentiated hiPSCs into chondrocytes avoiding EB formation. IPSCs were differentiated directly into mesenchymal SCs and then into chondrocytes that have the ability to repair osteochondral defects of arthritic joint in rat animal model [20]. Nevertheless, the creation of EBs and mesenchymal progenitors still required an additional step in differentiation of SCs towards chondrocytes.

The immunofluorescence analysis

The obtaining of stem cell-derived chondrocytes was confirmed by immunofluorescence (IF) (Fig. 2D,E) and immunohistochemistry (IHC) (Fig. 2F) analyses. As a chondrogenic markers, type II collagen, Sox9, Sox6 and chondroitin sulfate proteoglycans [22,30,42]. For the determination of pluripotency, the presence of transcription factors such as Nanog, Oct3/4 was studied. The expression of CXCR4 was also examined [13,22]. Moreover, the E-cadherin as an additional marker of undifferentiated cells was used. The presence of type II collagen and aggrecan is characteristic for hyaline cartilage, but not for fibrocartilage, which is responsible for failure in induction of a durable cartilage repair [47]. In our study collagen was expressed by all differentiated cells. Nevertheless, collagen in our cells did not create a such regular matrix structure like articular cartilage chondrocytes isolated from patients. The most intense expression was observed on cells surface after MC (TGF-β3 10 ng/ml) and EBs (TGF-β3 10 ng/ml and TGFβ1 10 ng/ml+ BMP-2 100 ng/ml). Sox5, 6 and 9 (SOX Trio) are the masters regulatory transcription factor taking part in chondrogenesis. Sox5 and Sox6 throughout cooperation with Sox9, induce production of type II collagen and aggrecan, which guarantee cell survival in precartilaginous condensations [44]. The highest and very specific expression of Sox6 and 9 we gained after differentiation through EBs in medium supplemented with TGF-β1 (10 ng/ml) and BMP-2 (100 ng/ml). Chondroitin sulfate together with collagen II is a cartilage-associated matrix molecules. Embryonic cartilage contains chondroitin-4-sulphate, whereas adult cartilage chondroitin-6-sulfate [32]. These important proteoglycans were present in all differentiated cells. The CXCR4 is characteristic for cells from mesodermal germ layer. The CXCR4 overexpression has been reported in primary chondrosarcoma tumors. The upregulation of CXCR4 is connected with its invasion and metastasis to the bone [30]. The presence of CXCR4 in all differentiated cells was observed, however the cells from MC culture revealed the expression of CXCR at the relative low level. This phenomenon can be interpreted in two ways. On the one hand, it can indicate the limited contribution of cells from mesoderm and consequently, the inefficient chondrogenic differentiation. But in this case, the expression of type II collagen would be observed at the lower level. On the other hand, it indicates safety of these cells. The low tumorigenic risk may be attributed to these cells. Cadherins are the large family of glycoproteins that play pivotal role in cell adhesion. E-cadherin maintains cell-to-cell contact in embryonic stem cells that grow as

individual colonies and positively regulate pluripotent signaling pathways in them [29]. N-cadherin is expressed during cellular condensation in the developing embryonic limb bud. The chondrogenic process is stimulated by Wnt3a, which increases BMP-2 mediated chondrogenesis throughout the N-cadherin-mediated adhesion [8]. In this case, we expected the decreased level of E-cadherin in comparison with hESCs. The immunofluorescence analysis confirmed our presumption except the cells after MC, that showed still the high expression of E-cadherin. The occurrence of chondrogenic process was corroborated by the considerable decreased level of pluripotent marker: Nanong and Oct3/4.The cells from PC confirmed that we obtained chondrocyte-like cells revealing chondrogenic markers. The chondrogenic process was the most efficient at 14 day because chondrogenic markers without necrosis was observed although the nuclear chondrogenic markers such as Sox6 and Sox9 were more visible at 21 day.

Conclusions

In conclusion, differentiation of hESCs into chondrocyte-like cells can be successfully achieved *via* EB formation, MC and PC. Chondrogenesis through EBs had the highest efficiency and was superior to MC and PC. The obtained cells show morphology characteristic of adult articular chondrocytes and expression of Col2, Sox6 and9, while the expression of pluripotency markers was significantly reduced. The chondrogenic process *in vitro* can be carried out both in serum-containing and serum-free chondrogenic media, providing the basis for chondrogenesis in accordance with GMP. For the chondrogenic process, the role of GFs is crucial, with the most powerful being TGF-β3, TGF-β1 and BMP-2.

Protocols were established to allow the relatively inexpensive, simple and highly efficient production of a large number of chondrocyte-like cells suitable for transplantation into the sites of cartilage injury, which constituted a step forward in repairing damaged articular cartilage. However, despite the strenuous efforts of numerous laboratories, the molecular mechanisms underlying the process of chondrogenesis are still poorly understood and require further investigation.

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